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Lymphocyte subsets and adhesion molecule expression in milk and blood of periparturient dairy cattle

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Abstract

Fifteen Holstein dairy cattle were monitored for lymphocyte subsets and expression of adhesion molecules on cells in milk and blood at parturition and at intervals up to 21 days post-partum. Using flow cytometry, we determined percentages of T cells (CD4+, CD8+, $\gamma\delta$) and B cells from milk and blood of these cows. We also measured expression of adhesion molecules (CD62L, LFA-1, LPAM-1, and CD44) on lymphocytes in milk and blood. Significantly higher percentages of CD8+ cells were found in milk than in blood at all time points while significantly higher percentages of B cells were found in blood than in milk at all time points. There were minimal to no significant differences in percentages of CD4+ or $\gamma\delta$+ cells between milk and blood. Expression of adhesion molecules was consistently higher on all subsets of milk lymphocytes compared with blood lymphocytes. These differences were most pronounced and statistically significant at calving and in the first week following calving. CD62L, LPAM-1 and CD44 were expressed on a significantly higher percentage of lymphocytes in milk at calving than in milk at subsequent sampling times, while LFA-1 expression on lymphocytes in milk was significantly lower at calving than at subsequent times.
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Keywords: Bovine; Adhesion molecules; Homing receptors; Lymphocytes; T cells; B cells

1. Introduction

Impaired immune function is a contributing factor to the high incidence of infectious disease observed in the periparturient dairy cow (Kehrli et al., 1989a,b; Kehrli et al., 1990; Kehrli and Harp, 2001). Decreased immune function not only increases susceptibility to new infections leading to such diseases as mastitis and metritis but also can allow sub clinical infections such as salmonellosis and paratuberculosis to become clinical (Kimura et al., 1999). This increased susceptibility to disease begins 2–3 weeks before parturition and extends into the first 2–3 weeks of lactation (Oliver and Mitchell, 1983; Smith et al., 1985; Oliver and Sordillo, 1988). Integral to determining the mechanisms of periparturient immunosuppression is an understanding of the factors that control the movement of leukocytes in and out of the mammary gland.

The influx of cells into the mammary gland and other tissues is controlled by a series of events involving receptor interactions between adhesion

Abbreviations: APC, allophycocyanin; LPAM, Lymphocyte-Peyers patch adhesion molecule; LFA, Lymphocyte function associated antigen; PE, R-phycoerythrin; PerCP, peridinium chlorophyll protein

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molecules (homing receptors) on leukocytes, and their recognition by complementary receptor molecules on vascular endothelium (Kraal and Mebius, 1997; Salmi and Jalkanen, 1997). It seems likely that increased expression of adhesion molecules would facilitate selective migration of cells from blood into mammary gland. Neutrophil infiltration following infection of the mammary gland is necessary for clearance of pathogens, but excess infiltration results in inflammation and tissue damage (Paape et al., 2000). Lymphocytes and macrophages also infiltrate the gland and along with resident cells in the gland, release cytokines that regulate the quality and intensity of the immune response (Nonnecke and Harp, 1989; Sordillo et al., 1997). A first step to understanding this regulation is to compare the expression of adhesion and homing receptor molecules that control the movement of lymphocytes from blood into the mammary gland. In the present study, we first determined the relative percentages of lymphocyte subsets (CD4+, CD8+, $\gamma\delta$+, and B cells) in milk and blood of cows beginning the day of calving, and at intervals during the first 3 weeks after calving. We then examined the expression of several adhesion molecules on these lymphocyte subsets. CD62L, or L-selectin, is a peripheral lymphocyte homing receptor that was initially identified in mice and humans (Gallatin et al., 1983; Lasky et al., 1989; Siegelman and Weissman, 1989) and subsequently in cattle (Bosworth and Harp, 1992; Walcheck et al., 1992; Bosworth et al., 1993). This molecule is critical to the initial step of rolling and tethering on vascular endothelium prior to extravasation into tissue (Springer, 1994). LFA-1 is one of the β_2-integrins, a family of adhesion molecules that mediate firm attachment of lymphocytes to endothelial cells following the initial rolling and tethering step (Kurzinger et al., 1981; Arnaout et al., 1988; Springer, 1994; Salmi and Jalkanen, 1997). Several of these molecules share a common β chain, CD18, bound to various α chains including CD11a to form LFA-1. LPAM-1 is another of the integrin family of molecules consisting of an α_4 and a β_7 chain. This molecule has been characterized as a mucosal homing receptor for lymphocytes (Holzmann et al., 1989; Holzmann and Weismann, 1989; Hu et al., 1992). CD44 is a proteoglycan that has been proposed to play a role in leukocyte trafficking to extra lymphoid sites of inflammation or as a nonspecific accessory adhesion molecule (Haynes et al., 1989; Aruffo et al., 1990; Bosworth et al., 1990; Denning et al., 1990; Koopman et al., 1990; Miyake et al., 1990). The expression of LPAM-1 and CD44 on bovine milk lymphocytes has not been previously reported. We hypothesize that by comparing the expression of these molecules on milk and blood lymphocytes at and shortly following parturition, we may begin to elucidate the relative contributions of lymphocytes from the various immune compartments to the immunologic repertoire during this critical period.

2. Materials and methods

Fifteen Holstein dairy cows were used in the study. All cows were first calf heifers and in good condition. The study period extended from calving to 3 weeks post-calving. Blood was collected from each cow by jugular venipuncture twice weekly beginning the day of calving until 3 weeks post-calving. Seventy-five μl of whole blood was added to individual wells of 96-well micro titer plates and red blood cells lysed by adding 200 μl of distilled water per well for 15 s followed by restoration of isotonicity with 20 μl of 10X phosphate-buffered saline (PBS). The plate was centrifuged at 400 × *g* for 2 min and the supernatant decanted prior to addition of antibodies as described below. Milk lymphocytes were prepared by collecting 50–150 ml of milk twice weekly from each cow beginning at calving until 3 weeks post-calving. Raw milk was centrifuged at 400 × *g* for 15 min and the cream layer was aspirated down to a total volume of 15 ml. The sample was vortexed, then diluted with 35 ml of PBS. The sample was then centrifuged as before and then aspirated to the pellet. The cell pellet was suspended in 2–3 ml of PBS and 75 μl aliquots were added to 96-well micro titer plates.

Milk and blood cells were labeled with anti-CD45 in all wells; further characterized by labeling with appropriate antibodies (Table 1) in order to identify B lymphocytes and T lymphocyte subsets, and then finally categorized as CD62L+, LFA-1+, LPAM-1+, or CD44+ by use of four color fluorescence flow cytometry (i.e. CD45, lymphocyte subset, and 2 adhesion molecules were examined simultaneously). Fifty μl of the appropriate primary antibodies were allocated to wells; plates were incubated at room

Table 1
Antibodies used for characterization of bovine lymphocytes

Antibody	Isotype	Specificity	Clone	Source
Mouse anti-bovine CD4	IgM	T helper	GC50A1	VMRD Inc. Pullman, WA
Mouse anti-bovine CD8	IgM	T cytotoxic/suppressor	BAQ111A	VMRD Inc. Pullman, WA
Mouse anti-bovine $\gamma\delta$ TCR	IgM	$\gamma\delta$ T cell	CACT61A	VMRD Inc. Pullman, WA
Mouse anti-bovine B cell	IgM	B cell	VPM30	Serotec Inc. Raleigh, NC
Mouse anti-bovine CD62L	IgG1	L-selectin	BAQ92A	VMRD Inc. Pullman, WA
Rat anti-mouse $\alpha_4\beta_7$-PE conjugate	IgG2a	LPAM-1[a]	DATK32	BD/Pharmingen, San Diego, CA
Mouse anti-bovine CD11a	IgG1	LFA-1[b]	BAT75A	VMRD Inc. Pullman, WA
Mouse anti-bovine CD44	IgG3	Hermes	BAG40A	VMRD Inc. Pullman, WA
Mouse anti-bovine CD45	IgG2a	LCA[c]	CACTB51A	VMRD Inc. Pullman, WA

[a] Lymphocyte–Peyers patch adhesion molecule.
[b] Lymphocyte function associated antigen.
[c] Leukocyte common antigen.

temperature for 15 min, then centrifuged at 400 × *g* for 2 min and decanted. One hundred μl of a cocktail of secondary antibodies (goat α-mouse IgG2a-FITC, goat α-mouse IgG3-PE, Southern Biotechnology Associates Inc., Birmingham, AL; rat α-mouse IgG1-PerCP, BD Immunocytometry Systems, San Jose, CA; goat α-mouse IgM-APC, Caltag Laboratories, Burlingame, CA) was added and cells were incubated as before, in the dark. Secondary antibody controls consisted of wells similarly processed, but without primary antibodies. All plates were then centrifuged, decanted and cells were washed 1X with PBS + 1% fetal bovine serum + 0.1% NaN_3, centrifuged, then resuspended in 100 μl of BD-FACSLyse to fix the cells and stored at 4 °C until data acquisition on a LSR flow cytometer (Becton-Dickinson, San Jose, CA). Data were analyzed using CellQuest software (Becton Dickinson). Milk and blood lymphocytes were gated on the CD45+ population, and then gated on the mononuclear cell fraction. Ten thousand events per sample were collected for blood lymphocytes and 5000 events/sample were collected for milk lymphocytes. Data for lymphocyte subsets are presented as the percentage of the total mononuclear cell population expressing each of the subset markers. Data for adhesion molecules are presented as the percentage of each lymphocyte subset co-expressing the indicated adhesion molecule. Significance of differences for each parameter between values for milk and blood at similar time points, and between values for either milk or blood at different time points was determined by comparing the means ± S.E.M. of data from all time points by one-way analysis of variance and Tukey–Kramer multiple comparisons test.

3. Results

Percentages of lymphocyte subsets in milk and blood were determined. Significantly ($P < 0.05$) higher percentages of CD8+ cells were found in milk than in blood at all time points. In addition, the percentages of CD8+ cells in milk at days 14, 18 and 21 after calving were significantly ($P < 0.05$) higher than at day 0, 4 and 7 (Table 2). In contrast, there were significantly ($P < 0.05$) lower percentages of B cells in milk than in blood at all time points

Table 2
Percentages of CD8+ cells in milk and blood

Day	# Sampled[a]	Milk	Blood
0	13	30.67 ± 3.46[b]	11.27 ± 1.12[c]
4	15	30.71 ± 2.92	11.31 ± 0.61
7	15	31.94 ± 3.30	11.14 ± 0.72
10	15	39.89 ± 3.48	14.99 ± 1.14
14	15	43.26 ± 4.11[d]	15.35 ± 1.04
18	15	46.69 ± 3.36	15.53 ± 0.84
21	12	49.02 ± 3.84	14.85 ± 0.68

[a] Number of cows from which milk and blood samples were collected.
[b] Data presented as mean ± S.E.M.
[c] Values were significantly ($P < 0.05$) different between milk and blood at all time points.
[d] Values for milk at days 14, 18, and 21 were significantly ($P < 0.05$) different from values for milk at days 0, 4, and 7.

Table 3
Percentages of B cells in milk and blood

Day	# Sampled[a]	Milk	Blood
0	13	7.75 ± 1.13[b]	25.38 ± 2.40[c]
4	15	4.65 ± 0.51	24.17 ± 2.18
7	15	4.84 ± 0.48	24.76 ± 2.70
10	15	5.75 ± 0.60	25.35 ± 2.33
14	15	5.98 ± 0.72	25.61 ± 2.08
18	15	6.43 ± 0.90	25.23 ± 2.49
21	12	5.67 ± 1.00	24.79 ± 3.33

[a] Number of cows from which milk and blood samples were collected.
[b] Data presented as mean ± S.E.M.
[c] Values were significantly ($P < 0.05$) different between milk and blood at all time points.

(Table 3). The percentage of $\gamma\delta$+ cells was 9–14% in blood and 12–27% in milk over the time course of the study. There was a significantly ($P < 0.05$) higher percentage of $\gamma\delta$+ cells in milk compared to those in blood at 18 and 21 days post-partum (22.70 ± 2.23 and 27.06 ± 3.94 versus 14.2 ± 0.78 and 14.13 ± 1.08, respectively). The percentage of CD4+ cells was 13–19% in blood and 16–25% in milk over the time course of the study. There were no significant differences in percentages of CD4+ cells, either between milk and blood at similar time points, or for milk or blood at different time points (data not shown).

Percentages of the different lymphocyte subsets expressing adhesion molecules were compared between milk and blood. All lymphocyte subsets expressed CD62L on a higher percentage of cells in milk than in blood (Fig. 1). Differences were statistically significant ($P < 0.05$) for CD4+ cells at days 0, 4, 7, 14 and 21; CD8+ cells at days 0 and 21; $\gamma\delta$+ cells at days 0 and 18; and B cells at all time points. Similarly, all lymphocyte subsets expressed LFA-1 on a higher percentage of cells in milk than in blood (Fig. 2). Differences were statistically significant ($P < 0.05$) for CD4+, CD8+, and B cells at all time points, and for $\gamma\delta$+ cells at days 4 through 21. All lymphocyte subsets expressed LPAM-1 on a higher percentage of cells in milk than in blood and differences were statistically significant ($P < 0.05$) at days 0 and 4 for all subsets (Fig. 3). Finally, all lymphocyte subsets expressed CD44 on a higher percentage of cells in milk than in blood (Fig. 4). Differences were statistically significant ($P < 0.05$) for CD4+ cells at days 0, 4, and 10; CD8+ cells at days 0 and 4; $\gamma\delta$+ cells at days 0, 4, 10, and 14; and B cells at all time points.

Changes were also seen in percentages of milk lymphocyte subsets expressing adhesion molecules

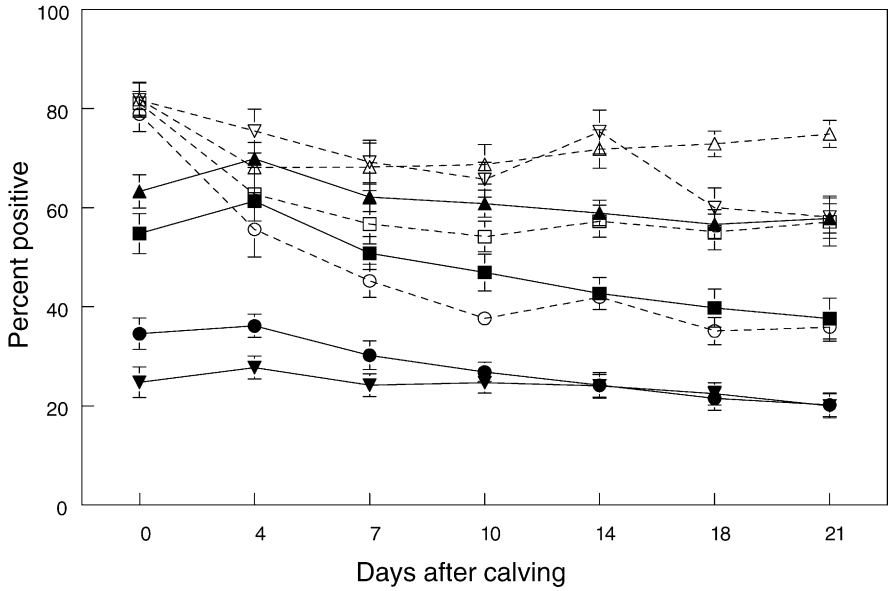

Fig. 1. Percentage of lymphocyte subsets expressing CD62L: (●) denotes CD4+; (■) CD8+; (▲) $\gamma\delta$ TCR+; (▼) B cells found co-expressing CD62L in blood. Open symbols of similar shape denote the respective subsets found in milk.

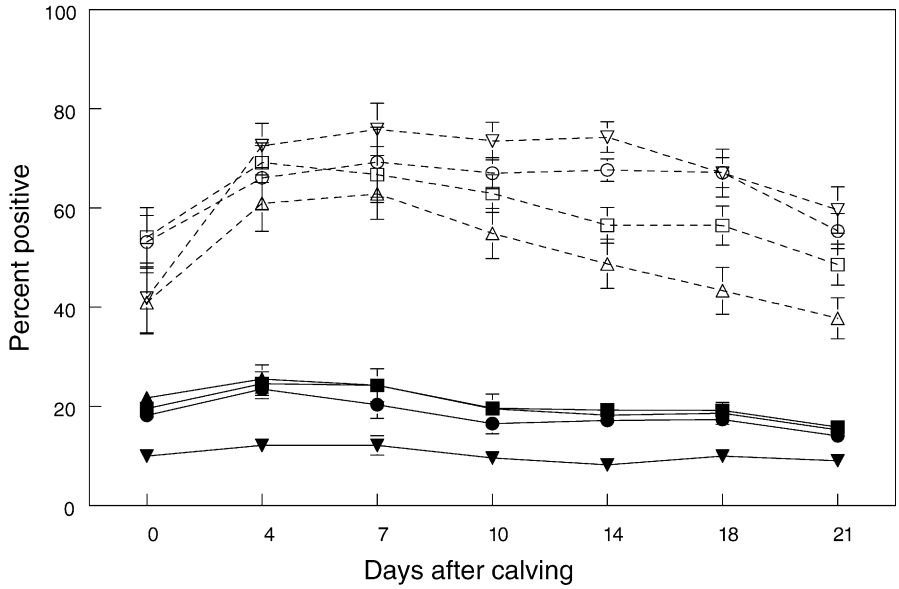

Fig. 2. Percentage of lymphocyte subsets expressing LFA-1: (●) denotes CD4+; (■) CD8+; (▲) $\gamma\delta$ TCR+; (▼) B cells found co-expressing LFA-1 in blood. Open symbols of similar shape denote the respective subsets found in milk.

over the time course of the study. CD62L, LPAM-1 and CD44 were expressed on significantly ($P < 0.05$) higher percentages of CD4+ and CD8+ subsets at days 0 compared to subsequent days (Figs. 1, 3 and 4). In contrast, significantly ($P < 0.05$) lower percentages of γδ+ and B cells expressed LFA-1 at day 0 compared to subsequent days (Fig. 2).

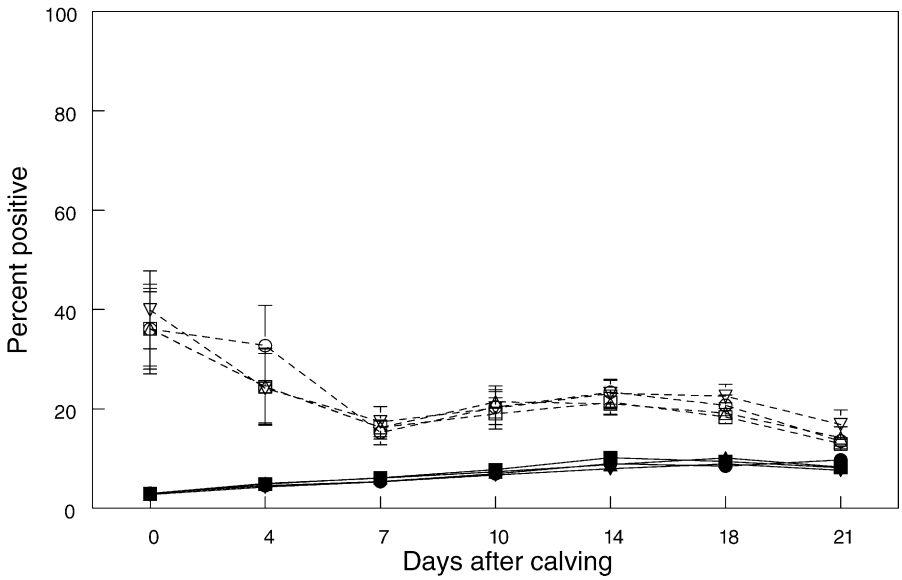

Fig. 3. Percentage of lymphocyte subsets expressing LPAM-1: (●) denotes CD4+; (■) CD8+; (▲) $\gamma\delta$ TCR+; (▼) B cells found co-expressing LPAM-1 in blood. Open symbols of similar shape denote the respective subsets found in milk.

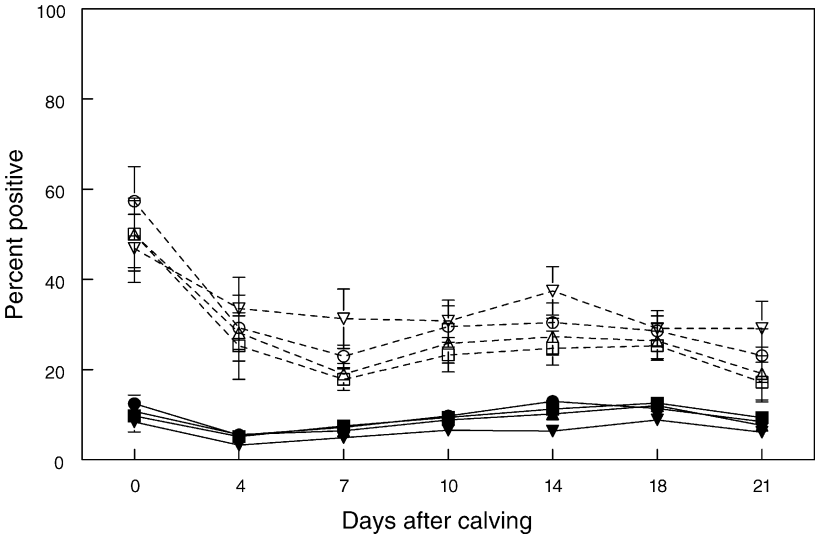

Fig. 4. Percentage of lymphocyte subsets expressing CD44: (●) denotes CD4+; (■) CD8+; (▲) $\gamma\delta$ TCR+; (▼) B cells found co-expressing CD44 in blood. Open symbols of similar shape denote the respective subsets found in milk.

4. Discussion

We found a consistent and statistically significantly higher percentage of CD8+ cells in milk compared with blood (Table 2). This is similar to previous findings (Park et al., 1992; Taylor et al., 1994; Shafer-Weaver et al., 1996; Van Kampen et al., 1999). Since bovine $\gamma\delta$ TCR+ cells may also express CD8 (Wilson et al., 2002) the interpretation of these findings is somewhat confounded; further study with dual color analysis would be necessary to resolve this. We also found a consistently higher percentage of $\gamma\delta$ TCR+ cells in milk from calving to 3 weeks post-calving, although the differences did not become statistically significant until 18 and 21 days after calving (data not presented). Van Kampen et al. (1999) reported a higher percentage of $\gamma\delta$ TCR+ cells in milk than in blood at calving, and then a lower percentage 3 weeks post-calving.

In the present study, B cells constituted about 25% of mononuclear cells in blood throughout, and 5–8% in the milk. These differences were statistically significant at all time points (Table 3). In previous studies using surface IgM as a marker for B cells, Concha et al. (1978) reported 45% B cells in blood and 20% in milk of mid lactation cows. Park et al. (1992) reported 20% IgM+ cells in blood and 10% in milk 2 days after parturition. Using a different B cell marker, Shafer-Weaver et al. (1996) reported 30% of lymphocytes in blood and 20–25% in milk expressed BAQ44A both post-partum and mid lactation. Thus, in the present and previous studies, there were consistently lower percentages of B cells in milk than in blood post-calving. Percentages of B cells reported in the present study were somewhat lower than previously reported values. We used an antibody (VPM30) that recognizes a 28 kDa molecule found on B cells in light, but not dark zone of lymph node germinal centers (Campbell et al., 1998). In addition, VPM30 recognizes activated T cells; up to 5% of intensely staining cells in freshly isolated peripheral blood mononuclear cells may be CD4+ (Campbell et al., 1998). Since percentages of B cells found in our study with this antibody are lower than previously reported values, it seems unlikely that inclusion of activated T cells in this population would be a factor. It is possible that, especially in milk, we are delineating a subset of B cells corresponding to the germinal zone B cells recognized by VPM30.

While we did not determine total cell counts for milk lymphocytes, previous studies have shown a precipitous decrease in cell concentration shortly before calving. This decreased concentration is maintained through early lactation (Jensen and Eberhart, 1981; Asai et al., 1998). Although a correlation

between decreasing cell concentrations and decreasing CD4+/CD8+ ratios was noted (Asai et al., 1998), interpretation of cell concentration data is confounded by the dilution effects of increased secretion during the immediate prepartum period (Jensen and Eberhart, 1981).

Measurement of adhesion molecule and homing receptor expression revealed several consistent patterns. First, expression of these molecules was consistently higher on all subsets of milk lymphocytes compared with blood lymphocytes. Second, these differences were most pronounced and statistically significant at calving and in the first week following calving. Thirdly, CD62L, LPAM-1 and CD44 were expressed on a significantly higher percentage of lymphocytes in milk at calving than in milk at subsequent sampling times, while LFA-1 expression on lymphocytes in milk was significantly lower at calving than in milk at subsequent times.

We found that CD62L was expressed on a higher percentage of CD4+ cells in milk than in blood, in contrast to a previous study (Van Kampen et al., 1999). However, in both studies, significantly higher percentages of CD8+CD62L+ and $\gamma\delta$ TCR+CD62L+ cells were found in milk than in blood on the day of calving. Expression of CD62L on B lymphocytes from periparturient cattle has not been previously reported. We found that a significantly higher percentage of B cells in milk expressed CD62L compared to those in blood at all time points (Fig. 1). On the day of calving about 80% of B cells were CD62L+. These data suggest that a high percentage of B cells trafficking into the mammary gland in the immediate periparturient period are coming from the peripheral lymphocyte pool. While the mammary gland is considered part of the common mucosal immune system in most species, in ruminants there is evidence that the peripheral immune system contributes more heavily to the immune repertoire of the mammary gland (Harp and Moon, 1987; Harp et al., 1988; Sheldrake et al., 1988). The present findings support this, and provide a rationale for parenteral rather than mucosal immunization of cows prepartum to generate antibody in colostrum to protect newborn calves against disease. These findings also support previous data showing that a high percentage of mammary lymph node lymphocytes express CD62L (Bosworth et al., 1993).

LFA-1 expression in milk was significantly higher than in blood for all subsets at all time points (Fig. 2). This difference was especially striking for B cells, approaching 80% LFA-1+ 1 week after calving. Since LFA-1 is an important accessory adhesion molecule that is necessary for efficient extravasation of lymphocytes from blood into tissue (Springer, 1994), the increased expression of this molecule on milk compared to blood lymphocytes may be related to increased recruitment of cells into the mammary gland following calving. One might speculate that the increase in LFA-1 expression on milk lymphocytes after calving may be in response to the reduction in expression of the other receptors in this study following the immediate post-calving period (Figs. 1, 3 and 4).

We found relatively low expression of LPAM-1 on lymphocytes in the blood; expression on milk lymphocytes was significantly higher for all subsets at days 0 and 4, but never exceeded 40% (Fig. 3). This is especially interesting considering that LPAM-1 is a mucosal homing receptor (Kraal and Mebius, 1997; Salmi and Jalkanen, 1997). The findings of the present study show consistently higher percentages of lymphocytes in milk expressing CD62L compared with LPAM-1 (compare Fig. 1 with Fig. 3) thus, further supporting the link of the mammary gland to the peripheral immune system in the bovine (Harp and Moon, 1987; Harp et al., 1988; Sheldrake et al., 1988). It is interesting to note that overall, LPAM-1 expression on lymphocytes was ~3-fold higher and CD62L was ~1.5-fold higher in milk compared with blood. Thus, while the overall percentage of lymphocytes in milk expressing CD62L was much higher than the percentage expressing LPAM-1 (62.37% and 22.18%, respectively), there does appear to be recruitment of LPAM-1+ lymphocytes into milk around parturition (in addition to recruitment of CD62L+ lymphocytes). This is consistent with the need of the neonate to receive a broad range of protection from infection in the immediate post-partum period.

The pattern of CD44 expression in our study was similar to that of LPAM-1; significantly more milk lymphocytes expressed this marker than did blood lymphocytes, especially in the immediate post-partum period (Fig. 4). This is consistent with the proposed role of CD44 as an accessory adhesion molecule for leukocyte trafficking to extra lymphoid

sites of inflammation or as a nonspecific accessory adhesion molecule (Haynes et al., 1989; Aruffo et al., 1990; Bosworth et al., 1990; Denning et al., 1990; Koopman et al., 1990; Miyake et al., 1990).

In summary, our results confirm and extend previous findings that there are significant differences in the expression of homing receptors/adhesion molecules on milk and blood lymphocytes after calving. Increased percentages of lymphocytes expressing adhesion molecules in milk compared to blood suggest that a migratory population of cells is being selectively recruited to the mammary gland from the circulation. The finding that twice as many milk lymphocytes express CD62L as LPAM-1 in the immediate post partum period is consistent with previous studies showing that the bovine mammary gland is more closely linked to the peripheral, rather than mucosal immune system (Bosworth et al., 1993; Harp and Moon, 1987; Harp et al., 1988; Sheldrake et al., 1988). Further investigation of mechanisms regulating infiltration of the mammary gland by lymphocytes may yield strategies to augment the immune response at the time of parturition, as well as to regulate damaging inflammatory responses to infections of the mammary gland.

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